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Review

Molecular basis for evasion of host immunity and pathogenesis in malaria

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Abstract

The article relates the ability of the malaria parasite *Plasmodium falciparum* to avoid a protective immune response, and to induce pathological changes, to the properties of specific parasite molecules. Cytoadherence and rosetting are important features of cerebral malaria and involve proteins located on the surface of the infected red blood cell. Proinflammatory cytokines, particularly tumour necrosis factor (TNF), play a role in protective immunity and in inducing pathology. Glycophosphatidyl inositol membrane anchors of parasite proteins possess insulin like activity and induce TNF synthesis. People subject to repeated infections in malaria endemic areas rarely develop complete or sterile immunity to malaria. They frequently carry small numbers of parasites in the blood, with little symptoms of the disease, illustrating a phenomenon termed semi-immunity. The basis for semi-immunity is incompletely understood. Malaria parasites are susceptible to several immunological effector mechanisms. The presence of extensive repetitive regions is a feature of many *P. falciparum* proteins. Available evidence suggests that the structural characteristics of the repeats and their location on the surface of parasite proteins promote immunogenicity. The repeats may help the parasite evade host immunity by (i) exhibiting sequence polymorphism, (ii) preventing the normal affinity and isotype maturation of an immune response, (iii) functioning possibly as B cell superantigens, (iv) generating predominantly thymus independent antibody responses, and (v) acting as a sink for binding protective antibodies. Sequence diversity in non-repetitive regions and antigenic variation in parasite molecules located on the surface of infected red blood cells also play a role in immune evasion. Some sequence homologies between parasite and human proteins may be due to molecular mimicry. Homologies in other instances can cause

Abbreviations: AMA, apical merozoite antigen; ATS, acidic terminal segment; CARP, clustered asparagine rich protein; CIDR, cysteine rich interdomain region; DBL, duffy binding like domain; DHFR-TS, dihydrofolate reductase thymidylate synthase; ELAM, endothelial leukocyte adhesion molecule; FIRA, falciparum interspersed repeat antigen; GBP, glycophorin binding protein; GPI, glycophosphatidyl inositol; HMS, hyperreactive malarious splenomegaly syndrome; ICAM, intercellular adhesion molecule; IL, interleukin; IFN, interferon; irbc, infected red blood cell; KAHRP, knob associated histidine rich protein; LFA, lymphocyte function associated molecule; LSA, liver stage antigen; MHC, major histocompatibility complex; MSA, merozoite surface antigen; *Pf*, Plasmodium falciparum; *Pf* EMP, Plasmodium falciparum erythrocyte membrane protein; *Pv*, Plasmodium vivax; rbc, red blood cell; RAP, rhoptry associated protein; RESA, ring erythrocyte surface antigen; S-antigen, heat stable polymorphic antigen; SERA, serine rich antigen; SICA, schizont infected cell agglutinin; TM, transmembrane; TNF, tumour necrosis factor; VCAM, vascular cell adhesion molecule

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autoimmune responses. The immune evasion mechanisms of the parasite need to be considered in developing vaccines. Protective immunity and pathology may be delicately balanced in malaria. © 1998 Elsevier Science B.V.

Keywords: Cytokine; Immunity; Immune evasion; Malaria; *Plasmodium falciparum*; Parasite protein; Pathogenesis

1. Introduction

Malaria is a re-emerging disease that produces 3×10^8–5×10^8 new infections and 1.5×10^6–2.7×10^6 deaths every year [1]. Much of the mortality occurs among children in sub-Sahara countries, with 4% of deaths among infants and 25% among 1–4 year old children in The Gambia being attributed to malaria [2]. The incidence is increasing in many tropical underdeveloped countries because of resistance of the parasite to common drugs, insecticide resistance in the mosquito vector, the effects of civil strife and organisational constraints. About half the malaria is caused by *Plasmodium falciparum* (*Pf*) and the balance by the three other species that infect man viz. *P. vivax* (*Pv*), *P. malariae* and *P. ovale*. *Pf* causes more deaths and has been more extensively studied because continuous in vitro culture of the asexual blood stages is possible [3]. This review therefore focuses on *Pf* malaria and reference is made to the other species only where relevant.

2. Life cycle of the parasite

Sporozoites are the infective forms of the parasite injected into the peripheral circulation when a *Plasmodium*-infected female *Anopheles* mosquito takes a bloodmeal. They travel through the bloodstream invading hepatocytes within 1 h of inoculation and undergo asexual schizogony to form merozoites. A dormant liver form of the parasite known as the hypnozoite is responsible for characteristic relapses seen in *Pv* and *P. ovale* infections. The hypnozoite is absent in the other two species of human malaria parasites. The rupture of infected liver cells releases merozoites into the bloodstream. These invade red blood cells (rbc) to produce ring stage parasites (so termed because of the signet ring like appearance when stained with Giemsa). The intracellular parasites are located within a parasitophorous vacuole formed during the invasion process. Rings synthesise proteases to degrade haemoglobin into amino acids. These and other nutrients obtained from the host enable the parasite to grow in 15–18 h into mature trophozoites. The trophozoite then undergoes schizogony to yield about 16 merozoites that are released by the rupture of the rbc membrane. The merozoites rapidly invade fresh rbc to continue the asexual blood stage cycle. An asexual rbc cycle takes 48 h in *Pf* and *Pv*. Some invading haploid merozoites develop into male and female gametocytes within the rbc. Gametocytes undergo gametogenesis in the mosquito midgut following ingestion. Fertilisation produces a diploid zygote that undergoes meiosis and differentiates into a motile ookinete. The ookinete traverses the midgut epithelium to lodge on the haemocoele side where it develops into an oocyst. Sporozoites produced in the oocyst then migrate to the salivary gland of the mosquito to continue the infection cycle (Fig. 1).

3. Pathology of malaria

Much of the pathology of malaria is caused by the asexual blood stages [4]. Fever due to falciparum malaria occurs initially at 48 h intervals and lasts for a few hours. Nausea, headache and chills accompany the fever. The fever follows shortly after the rupture of infected rbcs (irbcs), a process that is relatively synchronous at the initial stages of an infection. A rise in serum levels of tumour necrosis factor α (TNFα) in *Pv* infections precedes fever by about 30 min [5]. Anti-TNFα antibody reduces fever in Gambian children during *Pf* infections [6]. It is likely therefore that the release of parasite proteins and other components that occur during schizogony (Section 4) stimulates TNFα production by T cells and macrophages. TNFα, in turn, induces the release of interleukin-1 (IL-1), the endogenous pyrogen from macrophages and fibroblasts. Clinical complications

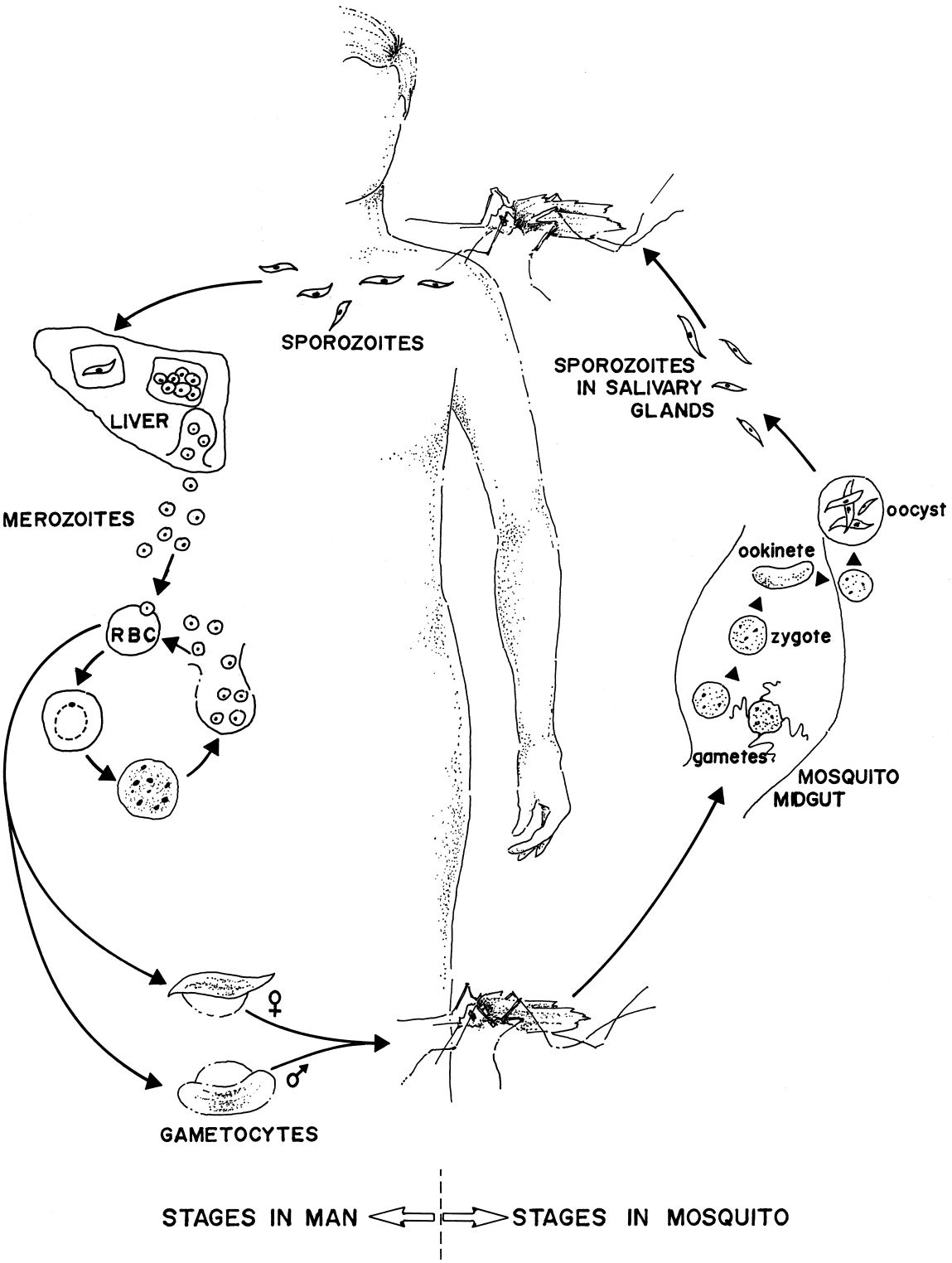

Fig. 1. Life cycle of a malaria parasite.

of *Pf* malaria occur particularly in non-immune adults and children who remain untreated for several days after the onset of fever. The most serious, and frequently fatal, complication is cerebral malaria. In severe cases this is associated with deep coma and generalised convulsions. Obstruction of cerebral venules and capillaries with trophozoites and schizonts is a characteristic histopathological finding in cerebral malaria. Mature *Pf* irbc specifically adhere to endothelial cells [7]. *Pv* on the other hand does not adhere nor cause cerebral malaria. Endothelial surface molecules reportedly mediating this cytoadherence include a platelet derived glycoprotein thrombospondin [8], intercellular adhesion molecule 1 or ICAM-1 [9], CD36 [10], E-selectin or endothelial leukocyte adhesion molecule 1 (ELAM-1) and vascular cell adhesion molecule1 or VCAM-1 [11], and chondroitin sulphate A [12,13]. The corresponding ligands on irbc surface are a family of 200–300 kDa parasite proteins termed the *Pf* erythrocyte membrane protein-1 or *Pf* EMP-1. These are coded for by 50–150 *var* genes [14–16]. The *var* genes may account for as much as 2–6% of the haploid genome [15]. Modifications to the rbc anion transporter band 3 glycoprotein, involving limited proteolysis, occur in mature *Pf* irbc [17,18]. A peptide that is only exposed on the altered band 3, composed of residues 546–553 of the human protein termed *Pf* alhesin, reportedly binds specifically to CD36 and therefore can contribute to cytoadherence of irbc [19]. Knob-like protrusions of the rbc membrane with deposition of electron dense material beneath the membrane are seen in electron micrographs of mature asexual stages of *Pf*. The knobs are frequently observed as the attachment points of irbc to vascular endothelium in brain autopsy sections from cerebral malaria patients [20]. At least two parasite derived proteins, a knob associated histidine rich protein or KAHRP [21] and a 315 kDa *Pf* EMP3 [22], are deposited in the knobs beneath the rbc membrane. These probably interact with the rbc cytoskeleton. *Pf* EMP-1 is anchored in the knobs by a transmembrane segment but contains an extracellular region that binds antibodies [14]. While the knob protrusions are needed for strong cytoadherence and consequent microcirculatory obstruction [23], strains of parasites that do not form knobs are also able to cytoadhere albeit more weakly [24].

Histopathological observations suggest that the binding of one or more uninfected rbc to irbc, a phenomenon termed rosetting [25], is also characteristic of irbc sequestered in the microvasculature [20]. Rosetting may be mediated by polymorphic 22–28 kDa proteins termed rosettins found on the irbc surface, with CD36 and A and B blood group substances on uninfected rbc serving as receptors [25]. It has also been argued that rosetting may involve the binding of C3b complement component on the irbc with the corresponding CR1 receptor on uninfected rbc [26]. More recently it has been reported that PfEMP-1 on irbc binding to CR1 on uninfected rbc is responsible for rosetting and that an allele of CR1 that demonstrates reduced affinity for PfEMP-1 is common in Africa [27]. Fibrillar material containing IgM or IgG and IgM are seen at the points of contact between irbc and uninfected rbc or endothelial cells but the significance of this phenomenon is not fully understood [28]. In a quantitative ultrastructural study of sequestration, the proportion of irbc and the tightness of packing of the cells in different tissues followed the order, brain > heart > liver, lungs and kidney > > blood [20].

Severe liver failure, circulatory collapse, acidosis, hypoglycaemia, anaemia, hyperpyrexia, acute pulmonary oedema and renal failure are other common clinical complications observed in untreated cases of acute falciparum malaria. Falciparum malaria is also a major cause of maternal death, abortion, stillbirth, premature delivery and low birthweight in endemic areas. The placenta is a preferred site for sequestration of irbc and a sub-population of the mature forms of *Pf* that adhere to chondroitin sulphate A in preference to CD36 can be found in infected placenta [29]. The increased susceptibility of pregnant women to malaria and the associated clinical complications may be due partly to this phenomenon.

Persons living in malaria endemic areas are often subject to repeated malaria infections and may develop chronic *Pv* infections. Such individuals often suffer from what is termed the hyperreactive malarious splenomegaly or HMS syndrome, which is characterised by persistent moderate or marked enlargement of the spleen [30]. This is accompanied by very high serum IgM levels, high antibody levels to parasite antigens, lymphocyte infiltration of hepatic sinusoids and liver enlargement, chronic anaemia and

pancytopenia. Susceptibility to secondary infections is increased in persons with HMS and this contributes to morbidity and mortality. The human spleen has an important role in immunity to malaria. Cytoadherence is believed to have been evolved by the parasite for avoiding the circulation of trophozoites and schizonts through the reticular cell/fibre filtration beds of the spleen, where they can be trapped and destroyed by the combined action of T cell derived cytokines and phagocytic cells. Trapping and destruction of mature iRBC in the spleen may be caused also by the inability of these cells to squeeze past the endothelial cells of the venous sinuses due to a decreased deformability of the rbc membrane [31]. Ring stages of *Pf* do not adhere and are capable of recirculating through the spleen. Therefore Giemsa stained blood smears of *Pf* patients show mainly rings while smears from *Pv* patients show mature stages besides rings.

4. A central role for TNF in malaria pathology and its induction by parasite molecules and IgE immune complexes

The importance of excessive production of TNF and other cytokines in causing malaria pathology was recognised several years ago [32]. TNFα and TNFβ are synthesised by macrophages and activated T helper cells of the T_{h1} subset. They cause the production of IL-1 by macrophages and fibroblasts which is in turn responsible for the characteristic malaria fever and the induction of IL-6 and acute phase proteins. Interferon-gamma (IFNγ) upregulates the expression of TNF receptors on a variety of cells [33], and its release by activated T_{h1} cells may potentiate the action of TNF in malaria. The expression of ICAM-1 on endothelial cells and its ligand LFA-1 (lymphocyte function associated molecule 1) on leukocytes is increased by TNF and this is directly related to the incidence of cerebral malaria in a mouse model [34]. One of the two alleles of the TNFα promoter region, associated with increased inducible and constitutive TNFα transcription, is significantly related to the susceptibility to cerebral malaria in Gambian children [35]. IL-10 produced by T_{h2} cells downregulates TNFα synthesis in falciparum malaria [36]. The ready activation of memory T_{h2} cells producing IL-10 may partly be the reason for the diminished malaria symptoms exhibited by many adults living in endemic areas.

Besides enhancing the sequestration of irbc in the brain microvasculature, increased TNF levels in malaria induces nitric oxide synthesis in endothelial and smooth muscle cells of the blood vessels. It has been suggested that nitric oxide produced in this way may diffuse into adjacent brain tissue and contribute to cerebral malaria and the other neurological changes observed in malaria [37].

The induction of TNF production in malaria is related to the release of parasite material during schizont rupture [5,6]. This material was shown to be heat stable and a phospholipid containing phosphatidyl inositol [38]. The active moiety has since been reported to be a glycophosphatidyl inositol (GPI) moiety that anchors many membrane proteins [39]. GPI anchors are present in the 185–200 kDa and 45 kDa merozoite surface antigens termed MSA-1 [40,44] and MSA-2 respectively [41–44], and a 76 kDa protease that is activated by the cleavage of the protein from the membrane anchor by a parasite-derived phosphatidyl inositol-specific phospholipase C [45]. A number of other parasite proteins also incorporate radiolabelled myristic acid into a putative GPI moiety from which the label can be cleaved by a *Bacillus cereus* phosphatidyl inositol-specific phospholipase C [44].

GPI attached to *Pf* merozoite surface proteins and purified GPI moieties can induce TNF and IL-1 production in macrophages [46]. They also produce a marked increase in triglyceride synthesis and glucose oxidation in isolated adipocytes, and a prolonged, TNF independent drop in blood glucose levels in mice [46]. Thus the purified GPI moieties appear to have strong insulin-mimetic effects that may be mediated through interference with normal host cell signalling pathways. Parasite material released during schizont rupture probably contains GPI precursors, protein associated GPI and free GPI produced by the action of phospholipase C. All these molecules may be involved in producing the described changes in the host.

Natural α1-3 galactosyl antibodies present in man and old world primates are reported to produce complement mediated damage to merozoites and thereby confer a degree of protection against *Pf* malaria [47].

Pf does not synthesise N-linked oligosaccharides [44,48] but does synthesise O-linked oligosaccharides [49] and GPI anchors for membrane proteins [44,50]. While there is one report that α1-3 galactosyl residues are absent in the GPI anchors of MSA-1 and MSA-2 [50], this needs to be confirmed in view of the observation that parasite glycolipids incorporate galactose [51]. Therefore the possibility that natural anti-α1-3 galactosyl antibodies also confer protection against malaria pathology by reacting with GPI moieties requires further exploration. In this context it is notable that antibodies to parasite molecules inducing TNF are found in malaria patients [52] and semi-immune Africans (reported in Ref. [53]). This parallels elevations in anti-α1-3 galactosyl antibodies seen in malaria patients and semi-immune populations [54].

More recently it has been reported that elevated blood IgE levels, comprising both non-specific and anti-malarial IgE, seen in persons living in endemic areas and in malaria patients are also responsible for overproducing TNF in malaria [55]. The proposed mechanism is the stimulation of TNF secretion by antigen–IgE immune complexes binding to the low affinity FcεRII receptor or CD23 on monocyte/macrophages [55].

Despite its central role in malaria pathology, the normal function of TNF in controlling parasite growth should be kept in mind. Thus TNF enhances neutrophil and monocyte/macrophage mediated phagocytosis and killing of asexual blood stages [56,57] and hepatic stages of *Pf* [58]. The fever produced as a result of TNF release may also help limit parasite growth [59].

5. Autoantibodies in malaria

Autoantibodies have been reported in malaria against single-stranded DNA [60,61], rbc polypeptides [62], cryptic rbc carbohydrate epitopes [63] and a variety of other tissues [64]. Many house keeping genes in the malaria parasite are homologous to the corresponding human genes. Sequence homologies may be expected to produce autoantibodies through bypassing T cell tolerance. This probably is the cause of the observed production of autoantibodies to the heat shock protein hsp70 in malaria [65]. There is extensive sequence homology between the parasite and human hsp70 [65]. At a different level, antibodies present in acute malaria patients that react with intermediate filaments of the cytoskeleton [66], may be produced against cross-reactive epitopes on the *Pf* MSA-1 protein [67]. There is no evidence yet to show that the rbc directed autoantibodies are wholly responsible for the severe anaemia seen malaria, which may be caused more by the rapid rbc turnover due parasitisation and erythropoiesis dysfunction in the marrow (reported in Ref. [68]). Similarly other autoantibodies have not yet been related to any specific pathological manifestations of malaria. This is an important area that requires more investigation.

6. Immunity to malaria

A single infection with a virus in many instances is able to produce solid immunity to reinfection for life. Smallpox was therefore eradicable. It is also possible to immunise against specific strains of viruses, e.g. the influenza virus, that demonstrate limited changes in the antigenicity of surface located molecules. Similarly, immunisation against bacterial toxins, eg. tetanus, or bacterial surface antigens, eg. from *Meningococcus*, can generate long-term immunity against infection. The situation regarding a protozoan parasite such as *Plasmodium* is less clear. Studies on individuals living in an area of continuous malaria transmission in Liberia show that the density of *Pf* in blood peaks between the ages of 6 months and 2 years and decreases after that with increasing age [69]. The incidence of malaria as assessed by stained blood smears also peaks at about this age, but then declines less sharply than parasite density with increasing age. However malaria morbidity, measured in terms of the mean body temperature produced by any given density of *Pf* parasites in the blood, declines with age [70]. The incidence of cerebral malaria and malaria-related deaths reaches a peak at age 4 year in The Gambia [70]. These and other earlier observations are frequently interpreted as evidence of a slow acquisition of immunity against malaria because of continuous or repeated exposure to the parasite. However studies on non-immune migrants to Irian Jaya and syphilitic patients who

underwent malaria therapy raise the alternate possibility that adults intrinsically acquire immunity more rapidly than children and that significant immunity may be developed even after one or few malaria infections [71]. It is clear however that immunity to malaria is never complete or sterilising in the sense that parasites are not totally eliminated from the circulation in many adults exposed to repeated infections in endemic areas. Rather the immunity appears to reduce parasite growth and disease symptoms in such persons, who may therefore be termed to be semi-immune to malaria. The differences between the immunity developed to viral and bacterial diseases and malaria may be attributed to the more complex life cycle and genome of *Plasmodium*. Consequently *Plasmodium* presents many more antigens to the immune system and demonstrates sophisticated mechanisms of immune evasion. The ability of the parasite to escape protective host immune responses tends to produce, in the absence of drug treatment, either chronic infections in the long term with minimal clinical symptoms (semi-immunity), or overwhelming infections that cause severe disease and death. However several immunological effector mechanisms operative against the malaria parasite have been elucidated and these are summarised below in Table 1.

7. Repetitive sequences of parasite proteins in immune evasion

7.1. Prevalence of repetitive sequences in Pf proteins

Many *Pf* antigens characterised to date contain regions composed of tandemly repeated sequences termed repeats. Tandem repeats, although found in collagen, serum complement proteins and associated membrane receptors, e.g. CR2 or CD21 [97], are relatively uncommon in human proteins. Because of the stability of epitopes in repeats and the presence of multimeric epitopes, it can be argued that the screening of *Pf* expression DNA libraries with immune human sera [98] selects for genes coding for antigens containing repeats. This may also be a consequence of a biased representation of antibodies directed against repeat region epitopes in immune sera. The circumsporozoite protein (CS protein) of *Pf* contains tandem repeats of the sequence NANP and the variant sequence NVDP [99]. Almost all the reactivity

Table 1

Parasite stage	Immunological mechanism	Ref.
1. Sporozoite	Antibody mediated inhibition of invasion of hepatocytes and parasite development	[72,73]
	C reactive protein and complement fixation	[74]
2. Infected hepatocytes	$CD8^+$ and $CD4^+$ cytotoxic lymphocytes kill infected hepatocytes directly	[75,76]
	Antibody dependent cell mediated cytotoxicity	[77]
	Indirect killing of infected hepatocytes via cytokines, etc. released by T cells and activated macrophages	[58,78,79]
3. Asexual blood stages	Agglutination of merozoites	[80,81]
	Blocking rbc recognition	[82,83]
	Complement mediated lysis of merozoites	[47]
	Antibody dependent killing by neutrophils	[56]
	Antibody dependent killing by monocytes/macrophages	[84,85]
	Killing by activated macrophage derived cytokines, reactive oxygen and nitric oxide derivatives	[86–89]
	Natural killer (NK) cell mediated cytotoxicity	[90]
	Reversal of cytoadherence	[91]
	Antibodies neutralising parasite toxins	[52,53,92]
4. Sexual stages	Transmission to mosquito blocked by antibodies to	
	(i) gametes	[93]
	(ii) zygote/ookinete	[94]
	(iii) mosquito midgut	[95]
	TNF mediated inactivation of gametocytes	[96]

against the CS protein in immune sera from endemic areas was reportedly directed against the repetitive epitope [100]. This indicates the immunodominant nature of the repeat region. A more recent study however shows that the conserved repeat region may be less immunodominant in areas of lower endemicity [101]. Natural antibodies to a heat stable protein found in the parasitophorous vacuole that is released during schizogony (termed the S-antigen) and a protein located on the inside surface of the rbc membrane in association with spectrin (termed ring erythrocyte surface antigen or RESA) also react predominantly with repetitive epitopes on the proteins [102].

Repeats are seen in many parasite proteins found in different sub-cellular locations and probably serving quite different functions. Apart from the examples discussed above, repeats are found in a 200 kDa protein localised in the parasitophorous vacuole of liver stage parasites termed LSA-1 [103], MSA-1 [40], MSA-2 [41–43], proteins associated with the rbc membrane knobs e.g. the knob associated histidine rich protein or KAHRP [104], and a protein present in the rhoptry–microneme complex of the parasite termed the rhoptry associated protein 1 or RAP-1 [105]. On the other hand, several *Pf* proteins including structural proteins, e.g. actin [106] and tubulin [107], rhoptry–microneme proteins, e.g. the apical merozoite antigen or AMA-1 [108], and enzymes, e.g. hypoxanthine-guanine phosphoribosyl transferase [109] and aldolase [110], lack tandem repeats.

7.2. Structural features of Pf repeats

The size of repeats in *Pf* proteins is quite variable. The clustered asparagine rich protein or CARP [111] contains long tracts of asparagine, while the serine rich antigen or SERA is composed of 11% serine present in polyserine tracts [112]. KAHRP and other histidine rich proteins of the parasite contain blocks of histidine [104]. Two allelic families of MSA2 are observed in laboratory [43] and field [113] isolates consisting of either 4–8 a.a. repeats rich in S,G and A or a 32 amino acid repeat and a ESNSRSPPITTT sequence repeated several times. A protein termed the glycophorin binding protein or GBP has multiple repeats of a 50 a.a. sequence [114]. Different allelic forms of some *Pf* molecules can possess repeat units of greatly varying sizes, e.g. in MSA-2. In two S-antigens there is underlying homology in the DNA sequences that produce different repetitive sequences that do not cross-react antigenically [115]. This suggests that these S-antigen repeats may have evolved from a common ancestral gene sequence. In contrast, the two known nonapeptide repeats of the CS protein of *Pv* show no homology at the DNA level and therefore could have evolved independently [116]. The 11 a.a. repeat at the 5′ end of RESA (sequence DDEHVEEPTVA) and the eight and four amino acid repeats in the 3′ end (sequences of EENVEHDA and EENV) are conserved among all known isolates [117]. Repeats within a particular region of a protein are often not exact, degeneracy being particularly evident towards the ends of each region. The genetic mechanisms for maintaining the repeats and for generating new repeat sequences are not known, but processes such as gene conversion, replication slippage and unequal crossing over are probably important. Immune pressure has been suggested to drive the formation of variant repeat sequences within a given protein (discussed in Section 9).

A comparison of the a.a. composition of the repeats containing fourteen or fewer amino acids and the non-repeat region of several *Pf* proteins shows significant differences [118]. Most marked is the paucity of cysteine and the hydrophobic amino acids Y, W, F, M, L and I in repeats. Analyses of ten individual antigens show that the repeats are more hydrophillic than the corresponding non-repeat regions of the proteins [118]. The difference between the repeat and non-repeat regions is less evident when large repeats $\geq$ 32 a.a. are used for comparison [118]. This may reflect a different role for the large repeats in parasite proteins. Chou–Fasman analysis of the secondary structure of the shorter repeats predicts that these probably assume a beta turn conformation [118]. Indeed circular dichroism studies indicate that the $(NANP)_n$ repeat of the *Pf* CS protein and the $(GQPQAQGDGANA)_n$ repeat of the *P. knowlesi* CS protein assume a reverse turn conformation in solution [119]. The amino acid composition and hydrophilicity of the repeats are also consistent with the formation of surface loops [120]. Loops and beta turns on the surface of proteins generally exhibit considerable atomic or segmental mobility and there

is a good correlation between such structures and the location of B cell epitopes in many proteins [121].

7.3. Possible functions of repeats in immune evasion

The presence of extensive repeats in many *Pf* proteins suggests a functional role for the repeats. It is possible that the repeats act as receptors or ligands for host proteins, with high affinity of interaction being generated through multivalent binding. Initially, this hypothesis was supported by an observation that the binding affinity of recombinant GBP to glycophorin is directly related to the number of repeats [114]. It was therefore thought possible that the GBP repeats have a function in the recognition of rbc by merozoites [114]. More recent work suggests that GBP is secreted into the rbc cytoplasm by mature parasites and that, while it may bind to intracellular domains of glycophorin, it does not play a role in rbc recognition by merozoites [122]. Repeat-host molecule interactions with a definite function have therefore not been unequivocally identified yet for *Pf* proteins. On the other hand there is evidence that repeats function to nullify a protective host immune response in different ways and some possible mechanisms are described below.

7.3.1. Antigenic diversity in repeats

Since repetitive sequences constitute immunodominant epitopes in parasite proteins, sequence variation in allelic forms of the molecules may prevent the population developing immunity to different strains of the parasite. The *Pf* S-antigen shows extreme diversity in repeat sequences and may provide an example. This is discussed in detail in Section 9.2.

7.3.2. Cross reactions between repeats interfere with affinity maturation of the antibody response

The use of immune sera and synthetic peptides demonstrates cross-reactivity between two forms of the eight amino acid repeats of the NF7 isolate S-antigen that differ in the use of R or L in one position [102]. Similarly immune sera and monoclonal antibodies (mabs) demonstrate cross-reactivity between the EENV and EENVEHDA repeats at the 3′ end of RESA and between EENV and the 5′ repeat of DDEHVEEPTVA [102]. Cross-reactions are also seen between epitopes in repeat regions and epitopes in non-repetitive regions of other proteins. Sequences recognised by an IgM mab that reacted with MSA-2, RESA, the FC27 isolate S-antigen and another asexual stage antigen composed of dispersed blocks of hexapeptide repeats with the consensus sequence PVTTQE (the falciparum interspersed repeat antigen or FIRA [123]) have been analysed [124]. This was done by reacting the mab with sequentially overlapping peptides from MSA-2 and FIRA [124]. The optimal epitope on MSA-2 had the tetrapeptide sequence STNS but a replacement net analysis revealed that a surprising degree of substitution was tolerated in each of the four positions [124]. The sequence STNS is found in the 32 a.a. repeat of the FC27 MSA-2 protein. The strongest mab binding peptides in FIRA were not found in the repeat but in other sequences that were rich in S, T and N [124]. Similarly, asparagine rich epitopes are reported to mediate the extensive cross-reactions, observed using polyclonal sera, between ten different asexual stage antigens and the $(NANP)_n$ epitope on the *Pf* CS protein [125]. Normal affinity maturation of the antibody response depends on the preferential binding of the declining numbers of antigen molecules with B cells whose antigen receptors have undergone somatic mutation in the V region to produce receptors with greater affinity for the antigen. This process takes place in the germinal centres of lymphoid organs. B cell blasts that are not selected in this manner in the germinal centres undergo apoptosis while the selected higher affinity cells differentiate into antibody secreting plasma cells or B memory cells. Isotype switching also occurs in the germinal centres under the influence of T cell derived cytokines. It has been argued that the presence of large amounts of antigens containing cross-reactive epitopes will allow continued stimulation of low affinity cells in germinal centres [102]. Since there is a limit to the numbers of B cells that can mature, imposed by physical constraints of the lymphoid organ, cytokine availability, etc., this effectively results in hypergammaglobulinaemia with a reduced proportion of high affinity antibodies and impaired isotype switching.

7.3.3. Repeats as B cell superantigens

A related possibility, for which there is at present no direct experimental evidence, is that the parasite repeats may bind to either framework Fv determi-

nants or the Fc portion of B cell antigen receptors thereby cross linking them and causing polyclonal B cell activation. Such a property would be analogous to retroviral proteins and bacterial exotoxins that act as T cell superantigens [126]. Consistent with this mechanism, polyclonal B cell activation, measured in terms of increased numbers of IgM, IgA and IgG secreting cells and higher levels of the corresponding immunoglobulins, has been reported in malaria [60]. The changes were more marked for falciparum than vivax malaria, and all the parameters tended to return to normal levels 15–30 days after chemotherapy [60].

7.3.4. Generation of a T-independent antibody response by repeats

Sporozoite immunisation experiments in mice that show that the antibody response to the $(NANP)_n$ repeats of the *Pf* CS protein is a T-independent one [127]. This is in contrast to the response to synthetic $(NANP)_n$ peptides where T dependence has been extensively documented. *Pf* proteins containing repeats may function as type 2 thymus independent antigens that are typically able to persist on macrophage surfaces and bind multivalently and cross-link antigen receptors on repeat-specific B cells. *Pf* proteins containing repeats may also have an element of type 1 thymus independent antigen activity. A parasite derived mitogenic activity, which may permit this, has been described [128]. T-independent antibody responses are usually short lived, predominantly composed of IgM and IgG3 and of low affinity. There is evidence to suggest that IgM dominates the natural antibody response to the *Pf* CS repeat in endemic areas [129]. Natural IgG antibody responses to MSA-2 in The Gambia are predominantly of the IgG3 subclass and directed principally towards the repetitive region [130]. Not all the repeat regions of different proteins however contain the extensive repeats seen in the CS protein, RESA, S-antigen or FIRA. If 12–16 haptenic groups are necessary for cross-linking B cell antigen receptors for a type 2 T-independent response [131], this condition is not met with in several proteins containing repeats e.g. MSA-1 and MSA-2. However immune complexes presented on the surface of antigen-presenting follicular dendritic cells may provide the required epitope density for such antigens to stimulate a T-independent antibody response.

7.3.5. Cross reacting repeat epitopes as an antibody sink

Another hypothesis regarding the role of repeats in immune evasion is based on the properties of surface location, segmental mobility and multimericity discussed in Section 7.2. Many proteins containing extensive repetitive regions, such as FIRA and the S-antigen, are present in relatively large amounts in schizonts and are released during schizont rupture. It has been suggested that they may, because of their inherent cross-reactivity with epitopes on functionally important merozoite proteins, serve to mop up antibodies that might otherwise bind to merozoites in the vicinity and prevent invasion [124]. Such a scenario may be particularly relevant in the microenvironment of a rosette of rbc surrounding a cytoadherent irbc in *Pf* infections.

The diverse mechanisms of immune evasion involving repeats discussed here are not mutually exclusive. Different repeats may generate different immune evasion mechanisms and it is possible that some repeats may have non-immunological functions such as binding to host receptors. Elucidation of V gene usage and somatic mutation patterns, histology of the germinal centres, conformation of repeats, the isotypes of natural antibodies to different repeats, and further characterisation of mitogenic activity in malaria may help to clarify some of the mechanisms discussed here.

8. Molecular mimicry and immune evasion

Many parasites mimic host molecules to avoid immunity. Schistosomes, which live in blood vessels of the host, readily absorb host serum components onto their surface thereby reducing immunogenicity. An analogous phenomenon has not been reported in malaria. Transforming viruses produce proteins, e.g. the SV40 T antigen, that interfere with cell cycle control in permissive host cells by mimicking the functions of certain host proteins. The sequences of two *Pf* proteins, termed Ag332 and Ag11-1, show sequence homology to α_1-thymosin, a thymic peptide hormone that modulates differentiation of T cells. Synthetic peptides based on the two *Pf* proteins had similar biological effects to the hormone on T cells

[132], and this may interfere with development of protective immunity in malaria.

9. Antigenic diversity as a mechanism of avoiding host immunity

Antigenic diversity for the purpose of this discussion is termed to be the existence of different allelic forms of parasite proteins. Since the parasite is haploid in man, only one allele per locus is expressed in the blood stages. The presence of *var* genes coding for the 200–300 kDa *Pf* EMP-1 protein on the rbc surface in multiple loci and the ability of the parasite to switch expression between different loci in the asexual blood stage [14–16] is termed antigenic variation and is discussed separately in Section 10. Sequence diversities in housekeeping genes of the parasite are probably no greater than that seen in the corresponding host proteins. The enzyme dihydrofolate reductase-thymidylate synthase (DHFR-TS) is probably the most extensively studied parasite housekeeping gene [133]. The sequences of DHFR-TS in different isolates of *Pf* are almost identical except for changes in the active site of the enzyme that generate resistance to the anti-folate drugs pyrimethamine and proguanil [133]. The situation in parasite antigens that play a role in host immunity is quite different. Using panels of mabs, extensive antigenic polymorphism has been shown in *Pf* [134] and *Pv* [135]. The basis for this polymorphism is the existence of multiple allelic forms of many proteins (reviewed in [136]) that have markedly different B cell epitopes. Reciprocal recombination within particular genes, that produce different combinations of B cell epitopes, adds to the diversity [136]. Furthermore within particular allelic forms there occur more restricted sequence changes such as point mutations which may give rise to further diversity in B cell epitopes and generate different T cell epitopes. Diversity in B cell epitopes is driven by antibodies. On the other hand diversity in T cell epitopes is more complex and involves selection during proteolytic processing, binding to MHC class I or II molecules, and recognition by T cell receptors. The structural nature and immunological significance of the diversity are best illustrated by considering three proteins in greater detail, viz. the CS protein, the S antigen and MSA-2.

9.1. CS proteins

The CS protein is the major constituent of the outer coat of the sporozoite. CS proteins from different *Plasmodia* show the same basic structure composed of a single central domain of extensive repeats (Fig. 2). Sequences that are relatively conserved between different species are found immediately to the N-terminus of the repeats and towards the C-terminus of the protein. Sequences in the conserved regions are reportedly involved in hepatocyte recognition [137]. The *Pf* CS repeat in all isolates is composed of the sequence NANP and the variant sequence NVDP. The *Pf* 7G8 isolate for example contains 37 × NANP and 4 × NVDP repeats [99] (Fig. 2). Two different alleles of the CS repeat are found in *Pv*, being composed of the nonapeptide sequences ANGA(G/D)(N/D)QPG and DRA(D/A)GQPAG [138]. An analysis of the frequency of occurrence of single nucleotide substitutions producing synonymous (d_s) and non-synonymous (d_n) a.a. changes in the *Pf* CS protein showed significantly greater non-synonymous changes in the region C-terminal to the repeats that is also known to harbour identified T_c and T_h cell epitopes [139,140]. Furthermore, this analysis showed that the non-synonymous changes in the C-terminus were more likely to be radical ones i.e. involving a change between the categories of positively charged (H, R, K), negatively charged (D, E) or neutral (all other) amino acids. Analysis of the T epitopes of *Pv* however produced conflicting data as to the significance of greater-non-synonymous a.a. changes [138,140]. Rodent malaria parasites on the other hand showed no greater d_n compared to d_s changes in the sequences containing known T epitopes [139]. It is possible that this is due to the fewer rodent CS protein sequences analysed. The relative

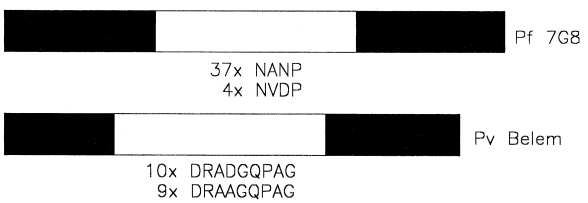

Fig. 2. Structure of the *Plasmodium falciparum* (7G8 isolate) and *P. vivax* (Belem isolate) circumsporozoite (CS) proteins. The alternative repeat found in *P. vivax* strains is ANGA(G/D)(N/D)QPG.

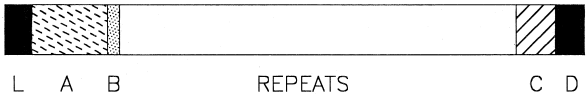

Fig. 3.

Serotype	Repeats
3D7	57×ED(E/K)VSNG(R/G)
HB3	80×ETGPGKAGEQG
Wellcome	65×GPNSDGDK
KF1917	78×GDQTEGS(S/A)GGK
NF7	43×A(R/L)KSDEAE
K1	22×GSDQEVKVQKEQ
V1	19×GGPGSEGPKGT
KF1916	100×AGSNE(E/K)
FC27	100×PAKASQGGLED

Structure of the S antigens with the sequences of the central repeats that determine the serotype. L – leader sequence; A – flanking region of limited variability; B – a non-repetitive sequence of 10–12 a.a. present in some proteins; C – a region present in some proteins that may consist of 15mer repeats; D – a region of limited variability [115].

conservation of the central repeat but variation in the T epitope regions is consistent with accumulating evidence that antibodies against the repeats are protective only at high concentrations that are not frequently seen in endemic areas [141], and that T cell dependent immune responses directed against infected hepatocytes (which may also express CS protein derived peptides on the surface) are important for immunity [141,142].

9.2. S-antigens

The S-antigen is a heat stable protein secreted into the parasitophorous vacuole [115]. It is released in copious amounts during schizont rupture and shows extreme size polymorphism (50–220 kDa) and serological diversity [115]. The function of the protein is unknown. Although a mab against the FC27 repeat reportedly inhibits parasite growth in vitro, there is no other definitive evidence as to its role in immune protection [143]. Nine S antigens have been characterised that differ in the sequence of up to 100 repeats in a centrally located domain [115] (Fig. 3). The sequence of the repeats determines the serotype of the protein, since human antibodies are directed primarily against epitopes in the repetitive region [102]. It is reported that over 50 serotypes may be prevalent in endemic areas [115]. However, the N- and C-terminal flanking regions are composed of a few defined and relatively conserved sequences. Analysis of the flanking regions for d_n and d_s showed that these were approximately equal, arguing against significant immune pressure for variation in these regions [140]. A similar analysis was not possible, or indeed required, for the central repeat because of the extreme variability. The rapid evolution of different repeats in the S antigen, accompanied by relative homogeneity of repeats within a particular molecule, probably involves mechanisms such as unequal crossing over, replication slippage and gene conversion. The extreme diversity of the S antigen repeats is good evidence for antibody driven selection. Support for this theory is provided by observations that individuals infected with one serotype are unlikely to be reinfected soon with the same serotype and that the prevalence of a serotype in a village population varies with time in a manner consistent with antibody driven selection [144,145].

9.3. MSA-2

The MSA-2 protein is an integral membrane protein on the merozoite surface and mabs to the protein can inhibit parasite growth in cultures [41,42,82]. Two allelic families of MSA-2 are present in laboratory and field isolates [43,113]. These differ chiefly in a central region containing repetitive sequences and flanking non-repetitive sequences, while the N-terminal 42 a.a. and the C-terminal 75 a.a. are conserved between alleles (Fig. 4). Within each family occur sub-families [43,146]. Limited recombination is observed between different alleles even within the central variable region [147]. Natural antibody responses are directed principally against the variable

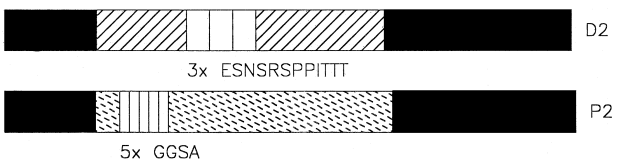

Fig. 4. Structure of two MSA-2 proteins from Sri Lanka. D2 and P2 belong to the two major allelic families represented by the K1/FC27 and 3D7 isolates respectively [113]. Conserved (black), allele-specific flanking (striped or stippled) and the repeat regions in the two molecules are indicated.

region of MSA-2 [130] but reactivity is also seen towards a peptide in the conserved C-terminus when a sensitive radioimmunoassay is employed [148,149]. Analysis of synonymous and non-synonymous mutations in MSA-2 revealed significantly higher non-synonymous changes in the N-terminus, but not in the more membrane proximal C-terminus, both within and between the two allelic families [140]. These changes, and the more obvious differences between the alleles in the repetitive region, are likely to be driven by selection pressure exerted by both antibodies and T cells. The D2 (Fig. 4) or FC27/K1 like allele is more commonly found in parasites isolated from patients [150,151] and is significantly more frequent in persons with symptomatic malaria [152]. The data therefore suggests that this allelic type may be more virulent.

Analyses, similar to those presented above for the CS protein, the S-antigen and MSA-2, showed that non-synonymous changes were significantly more frequent in some regions of genes coding for MSA-1 [153], the extracellular region of AMA-I [140,154] and all regions of a minor component of the sporozoite surface termed TRAP (thrombospondin related anonymous protein, Ref. [155]) [140]. Indeed two peptides independently shown to be T cell epitopes in MSA-1 [156] were located in a region where $d_n > d_s$ [140]. On the other hand d_n and d_s were approximately equal in the liver stage antigen LSA-1, the knob associated histidine rich protein KAHRP and, as described in Section 9.2, the S-antigen regions flanking the repeats [140]. The latter group of proteins/protein segments appear to be be evolving neutrally like pseudogenes i.e. without strong selection pressure either for conservation of sequence as in most house keeping proteins, or epitope diversification as with sporozoite and merozoite surface proteins.

A common explanation advanced for the age related change in clinical immunity to malaria [69] is that such protection requires significant immunity against conserved epitopes or a pool of common variant epitopes and that this in turn requires multiple exposure to infections. Antibodies to conserved epitopes on critical antigens, e.g. the 19 kDa C-terminus of MSA-1 [83,157], are indeed associated with protection against falciparum malaria. Since such antibodies are generally short lived [129,148–150,158], long term immunity in the absence of continuous reinfection, according to this theory, requires the development of sufficient numbers of high affinity B and T memory cells against critical epitopes on relevant parasite proteins.

10. Antigenic variation as a mechanism for evading host immunity

The location of asexual blood stages (except merozoites) and gametocytes of *Pf* inside the rbc shields them from damage mediated by antibodies directed against the vast majority of parasite molecules. Merozoites (and sporozoites) are exposed to antibodies for only a very short time before they invade target cells. Mature rbc do not express MHC molecules on their surface that are needed for recognition by cytotoxic T cells. Antigenic parasite derived molecules exposed on the rbc surface are however accessible to antibodies for relatively long periods. This is unfavourable to the parasite and would be selected against unless such molecules perform essential functions e.g. transport, or confer other advantages e.g. sequestration in capillaries. An antigenically variable surface antigen on the surface of irbc was first demonstrated in the monkey malaria parasite *P. knowlesi* [159]. The antigen was termed the schizont infected cell agglutinin or SICA since homologous antibodies agglutinated the irbc. The corresponding antigen identified later in *Pf* contained conserved and variable epitopes [160]. Consistent with one theory of age related develop-

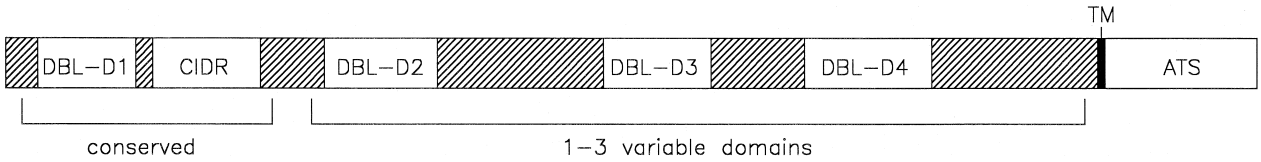

Fig. 5. Structure of the *var* gene products [15].

ment of immunity to malaria, children predominantly possessed antibodies to variable epitopes while adult sera showed greater reactivity with conserved epitopes on *Pf* SICA [160]. The SICA molecule undergoes rapid clonal variation in antigenicity without immune pressure in cultures (estimated at 2% per generation), with concurrent alterations in cytoadherence and rosetting characteristics [161]. The antigenic variation was associated with a large protein previously identified by radiolabelling of *Pf* irbc as *Pf* EMP-1 [162,163]. Although altered rbc components [17,18] and rosettins [25] may have a role in adherence of irbc, these molecules do not display the antigenic variability that is characteristic of *Pf* EMP-1. Genes for *Pf* EMP-1 have now been characterised and termed the *var* genes [14–16]. *Var* genes are present on different chromosomes and an estimated 50–150 genes code a family of 200–350 kDa *Pf* EMP-1 molecules that are predominantly located in the knobs on irbc. Expression of distinct var gene transcripts correlates with the synthesis of specific surface antigens on the irbc [16]. More than one var gene can be expressed in a parasite clone. Although the sequences of different *Pf* EMP-1 molecules are very variable, they contain certain common features (Fig. 5). One to four cysteine-rich domains homologous to regions found in the *Pv* merozoite ligand for Duffy blood group antigens [164] and the *Pf* merozoite ligand for glycophorin A [165] are present in *Pf* EMP-1 and these are termed Duffy binding like or DBL domains. The first domain near the N-terminus (DBL-D1) is the most conserved of the DBL domains. Between DBL-D1 and DBL-D2 is a relatively conserved cysteine rich inter domain region (CIDR) of 300–400 a.a. The relatively conserved nature of DBL-D1 and the CIDR at the N-terminus is consistent with a conserved function for the different *Pf* EMP-1 molecules. The numbers and location of the other DBL domains are more variable. A hydrophobic transmembrane segment (TM) is encoded in the 3′ end of the first exon and this is followed by a 45–55 kDa putative cytoplasmic acidic terminal segment (ATS) coded for by a second exon. A N-terminal signal sequence is absent from the deduced protein sequence of PfEMP-1.

It is speculated that conserved motifs in the DBL and CIDR domains maintain the conformation of the protein required for ligand binding and that the surrounding variable regions may be more exposed to antibodies and provide the basis for immune evasion [15]. Variations in the numbers and location of DBL domains D2 to D4 may govern the specificity of binding to the different cytoadherence receptors [8–13]. In an analogous manner to trypanosome variant surface antigens [166], different *Pf* EMP-1 molecules, despite extensive sequence variation, may retain a similar conformation that could be required for their function. Genomic rearrangements of the type seen in antigenic variation in trypanosomes are not essential for the expression of *var* genes and the mechanism of control of expression is yet to be determined.

11. Conclusions

Falciparum malaria has exerted significant selection pressure on human populations at the level of haemoglobin [167] and possibly major histocompatibility antigens [70]. *Plasmodium* is an ancient genus and malaria parasites are also reported to have influenced primate evolution 17–25 million years ago [47]. Such strong selection pressure implies that *Pf* malaria causes high mortality in untreated individuals and this is amply supported by existing evidence [2]. Semi-immunity to the disease only develops in those persons who survive the stringent selection process that normally occurs in endemic areas during childhood. The availability more recently of anti-malarial drugs, even in remote endemic areas, has however somewhat changed this classical epidemiological picture. It is not clear at this time what causes the semi-immunity (which is almost never sterilising immunity) observed in adult endemic area populations. Some possibilities are the acquisition of sufficient numbers of high affinity memory B and T cells against critical epitopes on parasite proteins, age and infection related changes in the lymphoid organs and down-regulation of the responses to cytokines mediating malaria pathology. These mechanisms are not mutually exclusive, and some changes may reduce fitness and render the individual, who is semi-immune to malaria, more susceptible to other infections. They therefore require further investigation.

Extensive resources have been devoted to producing a synthetic malaria vaccine in recent years. In

view of the complex and apparently effective immune-evasion mechanisms evolved by malaria parasites, and the semi-immunity observed in endemic area populations, it probably reasonable to ask the question whether the induction of sterile immunity against malaria is a realistic goal. Without a proper understanding of the causes and other effects of semi-immunity, sterilising immunity is perhaps the most desirable outcome of vaccinating against malaria. Immunisation with x-irradiated sporozoites of *Pf* and *Pv* is the one instance where sterilising immunity to malaria, although short lived, has been elicited in man [168]. Since using x-irradiated sporozoites is not practical for mass vaccination, attempts have been made to develop synthetic peptides, recombinant proteins, recombinant viral and bacterial vectors and DNA vaccines based on sporozoite and liver stage antigens for immunisation. The most promising synthetic sporozoite vaccine to date contains a part of the *Pf* 3D7 isolate CS protein fused to hepatitis B surface antigen (HBsAg) produced and assembled into particles containing non-recombinant HBsAg in yeast. The particles when injected three times into volunteers, with an adjuvant composed of monophosphoryl lipid A and a saponin, gave an estimated protection efficiency of 86% against challenge with 3D7 *Pf* sporozoites [169]. Monophosphoryl lipid A plus alum was less effective as an adjuvant in this clinical trial [169]. The stronger adjuvant that yielded the best protection in this study was reported to be well tolerated in vaccinees, but more detailed studies on its safety, and the protection afforded against other *Pf* isolates by the vaccine, are warranted before field use. Sterilising immunity against *Pf* blood stages has been reported in *Aotus* monkeys after immunisation with asexual stage parasites in Freunds adjuvant [170], but similar results have not been achieved with synthetic asexual stage vaccines incorporating adjuvants acceptable for human vaccination. It is possible that significant levels of proinflamatory cytokines are essential for developing sterile immunity. This may be simulated, for example, by injecting IL-12 which has been shown to protect rhesus monkeys against *P. cyanomolgi* sporozoites [171]. Malaria, and vaccination against malaria, may therefore illustrate a tenuous divide between protective immunity and pathogenicity in the human immune response.

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